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Potential Alterations in Immunogenicity by Combining or Simultaneously Administering Vaccine Components[a]

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One of the goals of the Children's Vaccine Initiative (CVI) is to reduce the number of contacts required to immunize a child fully. To meet this goal, new combination vaccines will need to be developed. Multiple new vaccines are under development that will be combined.[1] Currently, there are several licensed combination vaccines in the United States (see TABLE 1). These include mixtures of killed, inactivated, or nonreplicating vaccines (e.g., diphtheria-tetanus-pertussis [DTP], 23-valent pneumococcal polysaccharide vaccine) and live, attenuated replicating vaccines (e.g., measles-mumps-rubella [MMR] and oral poliovirus [OPV]). The licensed pneumococcal polysaccharide vaccine includes 23 pneumococcal serotypes. It is likely that combination vaccines for use in infant immunization programs in the United States will need to include the following vaccine antigens: DTP, poliomyelitis virus, hepatitis B (HBV), *Haemophilus influenzae* b (Hib), and probably pneumococcal and meningococcal polysaccharides (PS) presented as conjugate vaccines. In parts of the underdeveloped world, childhood vaccines will be required that induce protection also from tuberculosis and malaria.

Combination vaccines or the simultaneous administration of vaccines need to generate protective immunogenicity equivalent to their immunogenicity when administered separately. As combination vaccines or protocols are developed, they will be more closely evaluated than in the past. It is unclear whether the currently licensed 23-valent pneumococcal polysaccharide vaccine was studied as rigorously as would be done today and shown to retain an immunogenicity for each polysaccharide type equal to that of each when administered individually. Early studies suggested that mixing polysaccharides as a multivalent vaccine had no or minimal effect on immunogenicity.[2] Most importantly, the licensed 23-valent pneumococcal polysaccharide vaccine has been demonstrated to have protective efficacy, and any decrease in immunogenicity, if present, would be accepted based on its protective capacity.

Vaccines that are simultaneously administered today in the United States include

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TABLE 1. Licensed Combination Vaccines in the United States

- Diphtheria-tetanus-pertussis (DTP)
- DTP-HbOC (Tetramune™)
- OPV and IPV (3-valent)
- Measles-mumps-rubella (MMR)
- Pneumococcal polysaccharide (23-valent)
- Meningococcal polysaccharide (4-valent)

combinations of DTP, DTP-Hib, OPV/MMR, and HBV. The Centers for Disease Control (CDC) recommends simultaneous administration of DTP, OPV, MMR, and Hib in children if a subsequent visit is doubtful. Simultaneous administration has been found not to interfere with immune responses to: (1) DTP and OPV; DTP and IPV; (2) DTP and Hib polysaccharide vaccines; (3) MMR; (4) DTP, OPV, and MMR; and (5) pneumococcal polysaccharide and influenzae vaccines; (6) Hib conjugate vaccines and MMR, DTP, or OPV; (7) Hib, pneumococcal and meningococcal polysaccharide vaccines.[3,4]

Some recently developed combination vaccines have suggested interference with immunogenicity, while others have shown enhancement. In a trial in Chile (see Clemens *et al.*, this volume), antibody responses to the pertussis component of a DTP vaccine combined with an Hib conjugate (PRP-T) vaccine were depressed when the vaccine was administered simultaneously or separately.[5] In contrast, a combination vaccine (Tetramune™) composed of DTP with Hib conjugate vaccine (HbOC, HibTiter) was shown to be more immunogenic than separate, simultaneous injections of DTP and HbOC vaccine when administered at two, four, and six months of age[6] (see P. R. Paradiso, this volume). Higher antibody responses to all four components of the vaccine were observed. In the case of Tetramune, the increase antibody responses to the Hib capsular polysaccharide may be due to the presence of the whole-cell pertussis vaccine at the same site as the HbOC vaccine. Whole-cell pertussis vaccine has adjuvant-like effects[7] and can enhance the immunogenicity of other vaccine components, such as diphtheria toxoid. The explanation for the increased response to tetanus toxoid in the combined vs. separate vaccine is unknown, but may be related to the increased amount of immunogenic material at the site with the combination vaccine.

INTERACTIONS OF VACCINES

There are several possible causes for reduced immunogenicity of combination vaccines. These include physical or chemical interactions, interactions between live viruses, or immunological interference. Combination vaccines may have decreased immunogenicity of the individual components because of physical interactions among the vaccine components that affect stability, consistency, or immunogenicity. Buffers for one vaccine may not prove compatible with those of another vaccine. Adjuvants such as aluminum hydroxide and phosphate bind to inactivated vaccines by noncovalent ionic binding. Combination vaccines with adjuvants may result in some components of the vaccine that are not normally adjuvant-absorbed being presented with an adjuvant or being displaced from the adjuvant. Each individual vaccine component will need to be stably adsorbed to an adjuvant prior to mixing into a combination vac-

cine. There may be inherent incompatibility between vaccines. Some DTP and Hib vaccines contain thimerosal, which will destroy the potency of inactivated poliovirus vaccine (IPV). These vaccines, as reconstituted and stored today, cannot be mixed stably in a vial. Storage of the two components in dual-chambered syringes can circumvent this problem.

Combination live viral vaccines can interfere with each other. With live viral vaccines, local interferon production induced by one virus can inhibit replication of another virus. Simultaneous replication of polio and rotavirus vaccines in the gastrointestinal tract can interfere with their immunogenicity. When MMR vaccines were being developed, more immunogenic strains or higher viral doses were required to overcome interference of virus immunogenicity.[8] With OPV, there is some competition between virus strains (type 2 virus replicates faster than types 1 and 3), that does not occur with monovalent vaccine. This is compensated for by the administration of multiple doses of trivalent OPV. The current MMR vaccine shows no interference as formulated when administered either alone or with OPV.

The possibility of immunological interaction between different vaccine components of combination vaccines remains relatively unexplored. Interaction could either enhance or suppress the immune response to individual vaccine components. Overall, when tested, the theoretical possibility of enhanced reactivity or suppression of immune responses with vaccine combinations has rarely been observed. There are several theoretical possibilities by which immunizing with combination vaccines could lead to immunological interactions between vaccine components. These include effects on antigen capture, processing or presentation, or lymphocyte recognition and responses.

IMMUNOLOGIC INTERACTIONS–ANTIGEN CAPTURE, PROCESSING, AND PRESENTATION

Antibodies commonly recognize conformationally determined epitopes on antigens and can recognize either proteins or polysaccharides.[9,10] In contrast, T cells recognize epitopes of proteins that are presented as peptide epitopes on the surface of antigen presenting cells (APC) associated with class I or class II major histocompatibility complex (MHC) molecules.[11,12] Denaturation of B cell epitopes will destroy the ability of either serum or surface immunoglobulin to bind the vaccine antigen, so that conformational determinants will need to be maintained in designing vaccines intended to induce antibody responses. Polysaccharides cannot associate with MHC class I or II molecules. Class I and II molecules bind peptides generated inside the APC that are then transported to the surface of the APC, where they are recognized by T cells.[13,14] Preformed peptides can bypass this requirement for antigen processing. The T cell receptor, composed of α and β chains, recognizes the peptide epitope presented in the antigen-binding groove of the MHC molecule. CD4 on helper T cells (Th) interacts with MHC class II, and CD8 on cytotoxic T cells interacts with class I molecules, which lead to class II restricted and class I restricted responses being mediated by CD4+ T cells and CD8+ T cells, respectively.

CD4+ Th cells enhance the responses of B cells and other T cells leading to antibody production or elicitation of an inflammatory or delayed hypersensitivity response. CD4+ Th cells are stimulated by antigens presented by class II molecules and additional costimulation signals being mediated by the interaction of CD28 or CTLA4 on T cells with the B7 ligand on specialized APC-macrophages, dendritic cells, or acti-

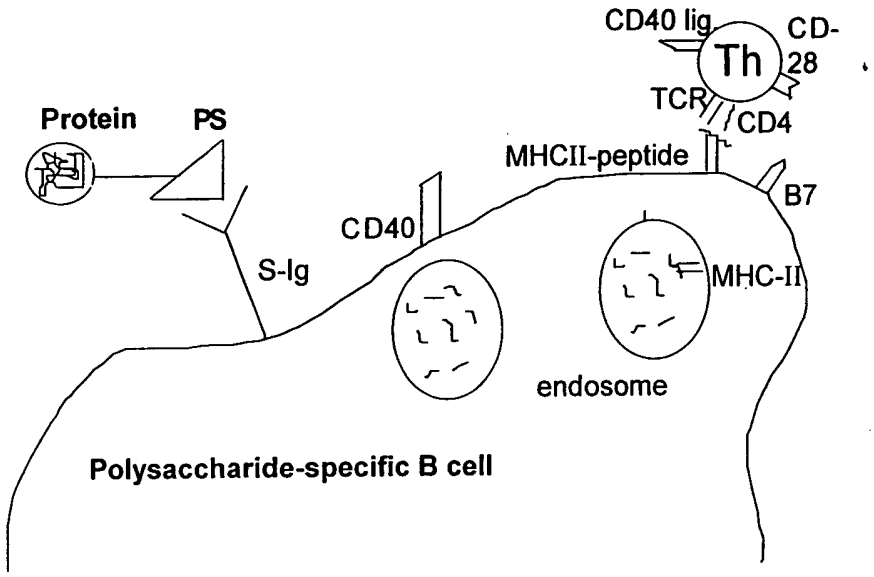

FIGURE 1. B cell processing and presentation of protein-polysaccharide conjugate vaccines.

vated B or T lymphocytes.[15] For resting B cells, an interaction between the CD40 ligand on activated T cells and B cell CD40 is required to upregulate B7 expression on the B cell. The B cell is stimulated to proliferate, differentiate, and respond to cytokines (IL-10, IL-4) that promote isotype switching and antibody secretion. CD8+ T cells are responsible for killing cells harboring pathogens or releasing lymphokines that activate cells to kill pathogens. Presentation of peptide-MHC in the absence of the costimulatory signal leads to T cell inactivation or anergy, which is associated with a block in IL-2 gene transcription.[16]

Different pathways are involved in MHC class I– and class II–dependent antigen presentation (see R. N. Germain, this volume).[14] Class I proteins must be present in the cytoplasm to allow the generation of peptides and their association with MHC class I molecules. Part of the life cycle of virus and some other intracellular pathogens reside in the cytosol, where they synthesize protein. The proteins are degraded in the cytosol and are then transported to the lumen of the rough endoplasmic reticulum, where they interact with MHC I. Class I peptides are usually precisely 8–10 residues long, have distinct allele-specific residue at certain positions, and are restricted to a limited number of peptides, in contrast to class II restricted peptides. To direct non-peptide vaccines into the class I pathway will require the use of live vectors that produce intracellular proteins or pH-sensitive, enzyme-sensitive liposomes.

MHC class II molecules are expressed on macrophages, dendritic cells, and B cells. Class II restricted peptides are generated in the APC following endocytosis of antigen bound to Fc receptors of the APC or surface immunoglobulin on B cells. The endocytosed antigen is then degraded into peptides that bind to class II molecules in the acidic environment of the late endocytic compartment. The class II peptide complex is then expressed on the surface of the APC, where it is recognized by Th cells. Class II peptides range in size from 12–24 residues in length and may have conserved motifs for particular polymorphic binding pockets of class II molecules.

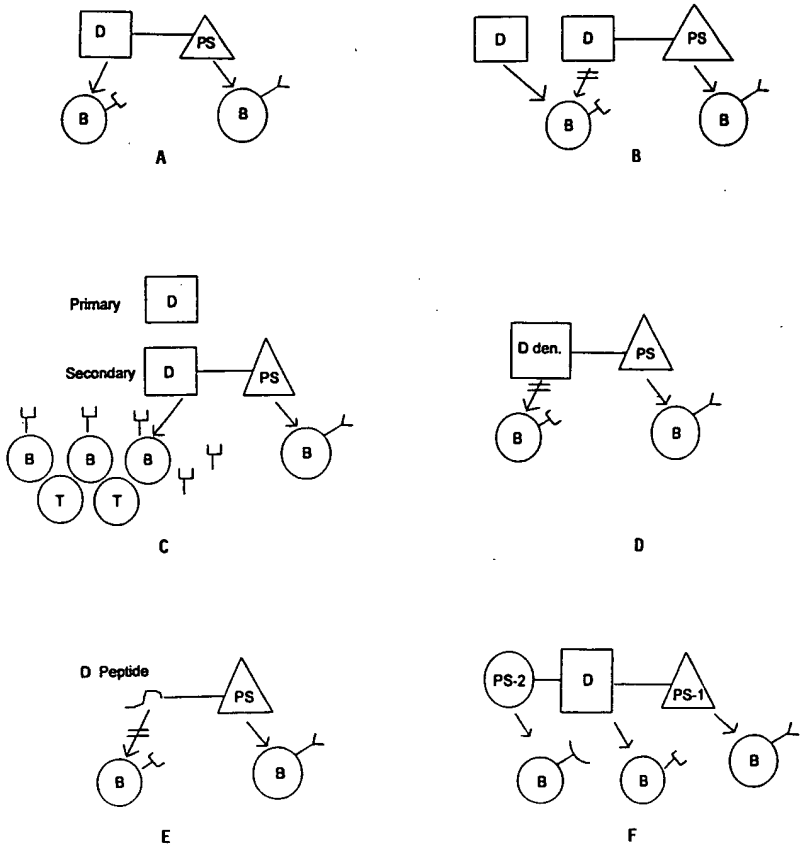

FIGURE 2. Competition between polysaccharide (PS)- and diphtheria toxoid (D)-specific B cells for binding to a diphtheria toxoid–polysaccharide conjugate vaccine.

Antigen at low concentration is preferentially endocytosed and processed by B cells expressing surface immunoglobulin (S-Ig) specific for that antigen through their preferential capture of antigen.[13,17] Carrier-hapten or protein-polysaccharide linked antigens, or so-called conjugates, are endocytosed by hapten- or polysaccharide-specific B cells that can then present carrier-specific peptides associated with MHC II to Th cells (FIG. 1). The carrier and hapten must be physically linked to allow their co-internalization. Naive T cells are first primed by antigen presented by dendritic cells and not resting B cells. Once primed, memory T cells can then be restimulated by antigen presented by a broader range of APC, including B cells.[18,19] Therefore, although B cells are efficient at antigen presentation, naive T cells require antigen presentation from a professional APC, with the best APC being dendritic cells.[20,21] In fact, naive virgin T cells are rendered tolerant if they are activated by B cells as the APC rather than by antigen presented by dendritic cells or macrophages.[22]

With carrier-hapten conjugates, there can be competition for antigen capture and presentation (FIG. 2A) between B cells with S-Ig specific for epitopes on the carrier protein and B cells specific for hapten.[17] Immunization with free carrier protein simul-

taneously with either a carrier-hapten or carrier-polysaccharide conjugate vaccine may prevent binding of the conjugate vaccine to carrier-specific B cells and redirect the conjugate to polysaccharide-specific B cells (FIG. 2B), leading to better antibody responses to the hapten. In addition, an increased number of carrier-specific Th cells may be induced. Preimmunization with the carrier protein may have divergent effects. Responses to a hapten or a polysaccharide presented as a conjugate immunization composed of the same carrier protein may be enhanced by an increase in the number and state of activation[19] of carrier-specific memory Th cells. On the other hand, carrier preimmunization can expand the number of carrier specific B cells and direct a conjugate away from hapten-specific B cells (FIG. 2C). This is one explanation[23] for carrier-induced epitopic suppression (CIES), as described below. Similarly, conjugates with low hapten density will also bias antigen presentation toward carrier-specific rather than hapten-specific B cells. Affinity of surface immunoglobulin may play a role in directing which B cell captures antigen if the concentration of antigen is low. Multivalent antigens, such as polysaccharides, may be able to compensate for the typical low affinity of antibodies to polysaccharide by being able to bind to multiple S-Ig molecules on the cell surface. It should be noted, however, that multivalent binding of antigens to S-Ig is not required for endocytosis of the antigen,[17] but may be required for full activation of the B cell.

Carrier-induced epitopic suppression is an immunological phenomenon in which antibody responses to haptens presented on a carrier are inhibited by prior immunization with the specific carrier.[24] The dose, route, choice of carrier protein, and presence of adjuvant dictates whether epitopic suppression or priming for responses to subsequent immunization with the carrier-hapten conjugate occurs. Suppression is induced in animals by large doses of carrier, antigen mixed with certain adjuvants, and generation of high titers of antibody to the carrier.[24-26] The mechanisms of carrier-specific epitopic suppression are not completely understood, but experimental models have implied a role for clonal expansion of carrier-specific B cells,[23] anergy of hapten-specific B cells,[27] antibody to the carrier protein, and suppressor cells[24,28] in its pathogenesis.

An increased number of carrier-specific B cells would act, as described above. It has been shown that hapten-specific memory B cells are induced in normal numbers with CIES but they are rendered tolerant, possibly from failure to receive adequate T cell help or having received an excess of suppressor activity when initially activated.[27] Serum antibody to the carrier protein has the ability to suppress antibody responses. Large amounts of anti-carrier antibody can inhibit responses to hapten-carrier conjugates. Although IgG antibody-antigen complexes can be bound by Fcγ receptors on APC and facilitate antigen presentation on follicular dendritic cells, antibody to epitopes can block antigen capture by epitope-specific B cells, inactivate the B cell, or affect antigen processing and presentation. Immune complexes may be shunted to Fc receptors on APC or to rheumatoid factor–specific B cells rather than to epitope-specific B cells.[29] Antibody to one epitope on a red blood cell (RBC) can suppress responses to other epitopes probably through signaling through both SIg and the FcγRII receptor on the B cell.[30] Immune complexes formed in antibody excess may not be processed in the APC in the same way as free antigen because antibody precludes attack by proteases.[31] It is clinically relevant that maternal antibody can interfere with primary tetanus toxoid, pertussis, diphtheria, or poliomyelitis immunization of infants.[32]

CIES becomes an issue in the choice of carrier proteins for combination vaccines composed of multiple (Hib, pneumococcal, meningococcal) conjugate vaccines or of slow release vaccines in which a chronic exposure to a carrier protein may occur. The relevance of these models for human immune responses, however, is not perfectly clear. To induce CIES in animals requires higher antigen doses on a weight-for-weight basis than is relevant for most human immunization. In infants, priming with DT vaccine can increase responses to Hib-DT conjugate vaccines,[33,34] although priming at too early an age may have no effect or be detrimental,[35] possibly secondary to induction of clonal anergy of immature carrier-specific T cells. Priming adults with DT in alum followed by secondary immunization one month later with a conjugate composed of DT linked to the Hib polysaccharide inhibited antibody responses to the polysaccharide.[36] Primary immunization of adults with a D-Hib polysaccharide conjugate vaccine followed by secondary immunization one month later with a T-Hib polysaccharide vaccine inhibited responses to the protein component of the vaccine.[36] This inhibition may be secondary to clonal proliferation of Hib polysaccharide–specific B cells that have the ability to preferentially capture the tetanus toxoid–Hib polysaccharide conjugate. CIES has been invoked with other human vaccines.[37,38]

Increasing the density of hapten on a conjugate,[23] priming with low rather than high doses of carrier protein,[25,26] removing B cell epitopes from a carrier protein,[25] denaturing the carrier protein (FIG. 2D), or using T cell carrier peptides devoid of B cell epitopes (FIG. 2E)[39,40] can prevent CIES. One approach to avoid CIES would be to use peptides composed of T-cell epitopes as carriers for vaccines. If the peptide was devoid of B cell epitopes, it would fail to stimulate antibody responses or expand carrier-specific B cells. Obviously it would be imperative that the peptide selected has the ability to bind human MHC class II antigens. There are, in fact, some peptides that may be universally immunogenic in man by being promiscuous in their binding to many different human class II alleles because they possess nested epitopes or possess one epitope that binds to conserved nonpolymorphic class II subsites.[41,42]

Interactions can also rise from competition at the level of antigen processing or transport to the surface with MHC II. It has been shown that polysaccharides (e.g., Ficoll, dextran) retained in macrophages interfere with antigen processing/presentation possibly through alteration in intracellular transport or lysosomal recycling mechanisms of MHC II peptide complexes.[43,44] Theoretically, large amounts of diverse polysaccharide-protein conjugates taken up by macrophages at the site of immunization could lead to inhibition in the capacity of the APC to present T cell epitopes.

Peptides can compete for binding to MHC-II and at the level of presentation to the TCR. Injection of immunodominant peptides with subdominant peptides can inhibit responses to the latter as shown with responses to hen egg lysozyme peptides in inbred strains of mice.[45] Dimerization of MHC class II molecules has been observed in crystals of MHC class II molecules.[46] MHC dimers would allow interaction simultaneously with two TCR complexes.[47] If correct, the same peptide may need to both be presented by class II molecules and engage the TCR, and, therefore, peptides could compete with each other at the level of presentation in T cells. In fact, there are peptide antagonists that are analogues of antigenic peptides with single amino acid substitutions that bind to the MHC class II molecule but fail to deliver signal 1 to the TCR.[48,49] They can block signaling from the native antigen presented on the same APC, possibly by lowering the affinity of TCR–class II-peptide interactions or blocking signal transduction.

IMMUNOLOGIC INTERACTIONS–ACTIVATION OF Th CELL SUBSETS

It has long been appreciated that cell-mediated immunity (CMI) and humoral immune responses are mutually antagonistic.[50] Immunization of mice with flagella with adjuvant induces antibodies without delayed hypersensitivity. If flagella is modified by acetoacetylation, CMI is induced with a decrease in antibody formation. If only low antigen doses are used for immunization, CMI is induced with no antibody formation. With high doses, antibody predominates; and with the highest doses, CMI again predominates. This antagonism or so-called "immune deviation" has a cellular explanation.

Helper T lymphocytes are composed of two distinct subsets designated T helper 1 (Th1) and T helper 2 (Th2) cells[51–53] (FIG. 3). The Th1 cells produce interleukin-2 (IL-2), interferon-γ (INF-γ), and tumor necrosis factor β (TNF-β), which activate macrophages to mediate the responses of CMI or delayed hypersensitivity. In contrast, Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and provide help to B lymphocytes for antibody production. In man, the difference in lymphokine production of the two subsets may be somewhat more quantitative rather than qualitative.[53,54] These functional subsets are mutually antagonistic, with each inhibiting the function of the other. Deviation towards a predominant Th1 or Th2 response can change the clinical picture of certain infectious diseases. In experimental *Leishmania major* infection, induction of Th1 cells induces production of IFN-γ, which activates nitric oxide production through induction of nitrate synthase in infected macrophage, which then kills the parasite and leads to recovery from infection.[55,56] If Th2 cells are induced, macrophage activation is inhibited by the IL-4 and IL-10 produced by the Th2 cells, and the parasites persist in the host, leading to death of the animal. Similarly, human visceral or cutaneous leishmaniasis is associated with increased IL-4 and IL-10 levels in serum or lesions associated with suppression of CMI and Th1 cell function.[57,58]

The mechanisms by which a naive Th cell deviates to become a Th1 or Th2 cell is related to cytokines that stimulate the naive T cell[59–61] (FIG. 3). IL-12, which is gen-

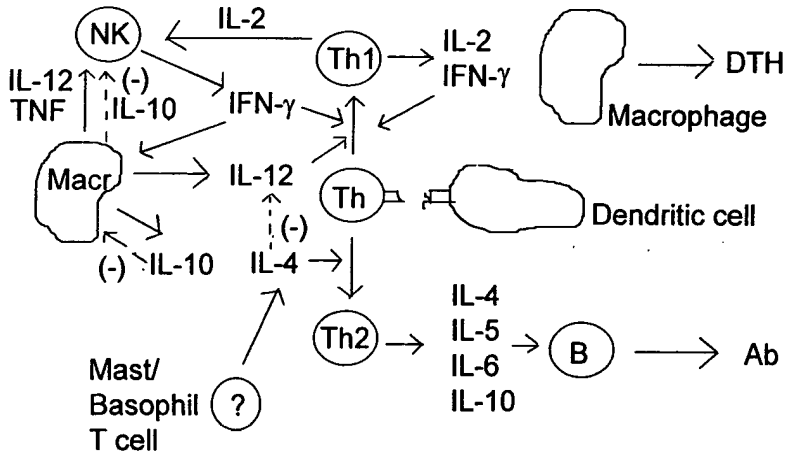

FIGURE 3. Activation of Th1 and Th2 cells.

erated from macrophages and B lymphocytes, stimulates production of Th1 cells.[62] Depletion of IL-12 blocks Th2 cell development and leads to Th1 cell development. IL-12 stimulates IFN-γ production from T cells and natural killer cells, and depletion of IFN-γ or IL-2 with monoclonal antibodies blocks Th1 and promotes Th2 development. Conversely, IL-4 promotes Th2 development, and can inhibit both IL-12 production by human monocytes and the ability of IL-12 to promote Th1 cell development.[63] Depletion of IL-4 inhibits Th2 and stimulates Th1 cell development, and IL-4 knockout mice fail to generate Th2-derived cytokines.[64] The source of production of IL-4 (?T cells, mast cells, or basophils) that stimulate Th2 expression is not clear. IL-10, which can also be produced by macrophages, can inhibit IL-12 (and TNF-α) production by macrophages, interfere with the stimulatory effects of IL-12 and TNF-α on natural killer (NK) cells, and block Th1 cell development.[65] The balance between IL-10 and IL-12 production by the APC may be critical in dictating which Th cell subset is expanded. The cytokine profile of the natural or nonadaptive immune response to antigen may, thus, dictate whether Th1 or Th2 cells are induced. The ability of several pathogens (*Mycobacterium, Listeria, Toxoplasma gondii*) to stimulate IL-12 production from macrophages correlates with their capacity to stimulate a Th1 response.[60,66]

In vivo T cell responses tend to exhibit either a polarized Th1- or Th2-like phenotype. This may be a result of the fact that each subset generates lymphokines that inhibit the development and function of the reciprocal subset. After T cell activation with both IL-4 and IL-12, the effects of IL-4 predominate with inhibition of Th1 cell development.[60,62] Initially, after macrophage phagocytosis there is early (4–8 h) release of IL-12 and TNF-α.[60] The latter is required with IL-12 for activation of NK cells for IFN-γ production. At 24–48 h after stimulation, the macrophage production of IL-10 peaks, which serves to down-modulate IFN-γ production. Preferential stimulation of one Th cell subset over the other is observed based on the type of stimulating APC (B cells stimulate Th2, and macrophages stimulate Th1 cells preferentially), the antigen dose, and the nature of the antigen. Viruses and intracellular bacteria promote a Th1 response.[66] Combination vaccines that predominantly stimulate IL-12 or IL-10 production by macrophages may deviate the immune response towards a dominant Th1 or Th2 cell response, respectively, because of the specific cytokines generated. The bulk of T cells producing lymphokines in a lymph node are not antigenically specific but are recruited into the site of immune responses.[67] The cytokines and cytokine inhibitors generated locally at that site will dictate the response in that local environment.

Active immune responses to one antigen may, therefore, theoretically interfere with immune responses to another simultaneously administered antigen. Mice infected with *Schistosoma mansoni* show a strong Th2 cell response that influences Th cell subset differentiation to unrelated antigens.[68] Similarly, in allergic individuals, purified protein derivative (PPD)–responsive CD4 T cell clones, which usually are predominantly type 1, produce both IL-4 and IFN-γ.[61] As new vaccines are produced and as new vectors are used to deliver multiple antigens at a single site, this balance between Th1 and Th2 responses will need to be considered. In addition, an antigen in a combination vaccine may elicit suppressor phenomena that could influence the immune response to other antigens. Antigen-specific T cells mediating suppression of antibody responses have been shown in animal studies.[69–71] The exact mechanism and cells that mediate suppression remain controversial. T suppressor cells may function by killing, inactivating, or releasing soluble mediators that inhibit T cells, B cells, or APC. Bloom and colleagues have shown that patients with leprosy generate CD8+ T suppressor cells that secrete IL-4 and anergize lepromin-stimulated CD4+ T cells.[70,71] They suggest that

there exists both CD4+ and CD8+ type cells that can suppress B cells, and CD4+ and CD8+ type 2 cells that can suppress delayed type hypersensitivity.

IMMUNOLOGIC COMPROMISES IN DEVELOPMENT OF COMBINATION VACCINES

A decrease in immunogenicity of combination vaccines may be acceptable as long as protective efficacy of the vaccine is preserved. It may not always be possible, however, to perform controlled clinical efficacy trials. Serologic assays that correlate with efficacy will need to be used as surrogates for efficacy trials. Combination vaccines will need to induce those antibody titers that correlate with clinical protection even if the magnitude of the response is not as great as that observed with the individual vaccine. One will need to keep in mind that the ultimate goal of the CVI is to protect children from infections with a limited number of encounters with health care providers. Combination vaccines with less than maximum immunogenicity may have to be accepted to meet these goals.

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